

Isolation and Characterization of *Bacillus anthracis* Hfq P

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Introduction:

All cellular species of life undergo the same order of transfer of genetic information from DNA template to messenger RNA(mRNA) by transcription followed by translation of mRNA to protein. This apparent simplicity is modified and regulated by other processes that interfere, enhance, and add on to this order. One such example is the regulation of the structure and function of mRNAs by small non-coding RNAs and protein Hfq. Hfq, first found as a *host factor* of *Escherichia coli* necessary for the replication of RNA bacteriophage $Q\beta$ -RNA¹, is a protein of 6 monomers of approximate 11.17 kDa for a total of about 66.6 kDa in molecular weight. The hexamer can bind to single stranded QB RNA containing some double stranded regions². Further studies discovered that mutant Hfq resulted in pleiotropic phenotypes such as decreased growth rate and numbers, increased cell length, and sensitivity to UV³. Comparison of *E. coli*'s Hfq's sequence with other bacterial genomes showed the protein appeared to be conserved widely in bacterial species^{4,5}. Homologs of Hfq also exist in archaea and eukaryotes⁶.

Hfq appeared to have broad effects by regulating RNA which influenced the translation of specific genes. For instance, Hfq with the help of sRNA increases the stability of rpoS mRNA transcripts for translation into the protein rpoS sigma factor needed for transcription of other genes responsible for environmental stress responses^{7,8}. The significance of rpoS lies in its activation of about 50 genes, some responding to starvation, osmotic shock, acid shock, heat shock, and cold shock⁹. In other instances, Hfq destabilizes mRNA such as in the case of mutA and transcripts of Pmiaa and P1hfqHS. Since Pmiaa and P1hfqHS are promoters of Hfq, this suggests that Hfq can autoregulate itself when it becomes too numerous¹⁰. Hfq also helps

poly(A) polymerase I compete against exoribonucleases to elongate poly(A) tails of transcripts. In vivo, the lack of Hfq reduced the lengths of the poly(A) tails, which are a requirement in bacteria for successful translation to protein. Similarities to the PABPII poly(A) binding protein, responsible for mammalian RNA poly(A) elongation, suggests comparable systems of poly(A) synthesis in both eukaryotes and prokaryotes ¹¹.

Recently Hfq has been gaining more attention due to its connection to bacterial virulence in pathogens such as *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Shigella flexneri*, and *Listeria monocytogenes*^{12,13,14,15}. Hfq also possesses a bacterial community-wide effect through its regulation in quorum sensing, a system of chemical messengers to other bacteria, in *Vibrio cholerae*¹⁶. This implies that Hfq not only directs intracellular activities but also extracellular to impact other bacterial cells in the environment and community. Interestingly, the lack of Hfq may hold some benefits for the cell such as increase in envelope biogenesis in *Salmonella enterica*¹⁷.

To date there has been limited research for the Hfq P, one of two Hfq copies found in *Bacillus anthracis*⁴ and the target of this paper. One recent study looked into *Bacillus subtilis*; however it only showed that its Hfq was not necessary for the stabilization of their RNA of interest: the SR1 which regulates arginine catabolism¹⁷. Thus it is valuable to determine the importance of Hfq P in the *B. anthracis*, a bacteria that could be used for bioterrorism.

Here, we will outline the isolation and characterization of this Hfq P. We hypothesize that the Hfq P is similar in biochemical properties to the *E. coli* Hfq. If true, further study on this Hfq P may reveal its potential as a target for medicine and treatment.

Methods:

Hfq P gene, Ligation to PTYB11, and ER2566 Transformation with pba Hfq P

The DNA coding region for the *B. anthracis* Hfq P was produced by PCR and given to the lab by Dr. Saleem Khan from the University of Pittsburgh. The gene was then modified for cloning by PCR, creating a Sap I site and SmaI site at the beginning and end of the gene, and ligated into a pTYBll plasmid (from New England Biolabs). This new plasmid, now called pba Hfq P, is credited to Dr. Roger Wartell and Nabil Wilf.

We transformed ER2566 *E. coli* cells (from New England Biolabs) with the pba Hfq P plasmid. Competent ER2566 cells mixed with pba HfqP in a chilled Falcon 2059 tube were incubated in ice for 30 min, heat shocked for 42°C for 90 seconds, and transferred back into ice for 2 minutes. Afterwards, the cells were shaken at 37°C for an hour in SOC media then plated onto LB/amp (100µg/mL) plates for overnight growth. The pba Hfq P was isolated from these transformed cells using instructions from the Promega Plasmid Mini-prep kit for verification of a complete and intact Hfq gene.

Isolation of Hfq P and E. coli Hfq by IMPACT-CN

IMPACT-CN intein-chitin column protein isolation procedure was used to obtain both the *E. coli* Hfq and *B. anthracis* Hfq P. Both isolations were nearly identical; transformed ER2566 cells were grown to .6-.8 OD (600 nm) and then incubated at 16 °C overnight with the addition of 40 mM of IPTG for Hfq monomer and intein induction. Cell lysis done with 13 rounds of 10 pulses in ice at 35% duty cycle and output at 4, by VWR Branson Sonifier 450 or by French Cell press at 800 psi for a total of 8 rounds. The column was washed with 1 M NaCl, 20 mM Tris pH 8.5, and 1 mM EDTA and cleaving buffer at 40 mM DTT, .5M NaCl, 20 mM Tris pH 8.5, and 1 mM EDTA. The eluate can be purified of DTT and reduced in volume by either centrifugation (30 kDa MW filter for *E. coli* and 10 kDa and 5 kDa MW filters for *B. anthracis*) or by dialysis (3.5 kDa filter) and lyophilization. Subsequent dialysis or centrifugation

was used to clean the sample from degraded nucleic acid from the micrococcal endonuclease digestion step. Micrococcal endonuclease treatment with 5-7 units/mL, 5mM CaCl₂, .3 M NaCl, 20 mM Tris for an hour was performed to reduce the nucleic acid contamination seen by the spectrophotometer. Isolation of proteins were heavily assisted by Taylor B. Updegrave.

Polyacrylamide Gel Electrophoresis

Both native and SDS denaturing gels used Precise 4-20% gradient polyacrylamide precast gels with Tris-HEPES buffer with Prosieve Protein Ladder (5K to 225 K MW, 550µg of protein ladder/500µL). Loading buffers either included bromophenol blue or xylene cyanol. Samples for the denaturing gel contained SDS and was boiled for 5 minutes. Gels were either stained with Coomassie Blue or Sypro Orange (used manufacturer's instructions), destained with 7.5% acetic acid, and photographed by Alpha Innotech Corporation MultiImager Light Cabinet and ImagerX.

Results:

We hypothesized that the physical properties of the *E. coli* and *B. anthracis* Hfq P hexamer would behave in a similar manner. Because of our hypothesis, we isolated the *B. anthracis* Hfq P based on the success in *E. coli* Hfq's isolation and purification. In Figure 1, Lanes 2 and 3 show induced cells with a band corresponding to the *E. coli* Hfq monomer and intein tag. Lane 4 shows no band due to the monomer-intein binding to the chitin column. In Figure 4 (A), Lanes 4,5,6,7 all show induced cells with the *B. anthracis* Hfq P monomer-intein and Lanes 8 and 9 lacking this distinct band to represent monomer-intein binding to the column. Lanes 2 and 3 are uninduced cells serving as a control to ensure that IPTG was necessary for

induction. These gels ensured us that the induction and isolation by chitin column was done correctly.

After the eluate was collected (about 70 mL) the *E. coli* Hfq was centrifuged with a 30 kDa MW filter to remove the DTT and reduce the volume of eluate. In Figure 2, the spectrophotometer indicated that there was nucleic acid contamination as well as our target protein, as seen on the graph as a high absorbance around the 260 nm area and 280 nm respectively. A pure protein sample would have a ratio of 1.7 between the maximum and minimum absorbance at 270 nm and 250 nm. After nuclease digestion, the Hfq sample had a better ratio about 1.5 (composed of the much lower absorbance at 250 nm compared to the one at 270 nm). A native protein gel indicated that there was hexamer; however the gel shows a smear at 100 kDa MW (Figure 3). This was a peculiar result, because the *E. coli* hexamer is only 66.6 kDa MW.

We were successful in isolating Hfq P from the chitin column as indicated by the strong bright bands and its disappearance in Figure 4, however centrifugation with a 10 kDa and then a 5 kDa MW filter had lost the hexamer. To eliminate this problem, we turned to a gentler technique: dialysis with 3.5 kDa MW filter bags and lyophilization. After dialysis, the Hfq sample's spectrophotometer readings also indicated heavy nucleic acid contamination, and a nuclease digestion followed. The second dialysis was performed to remove the degraded nucleic acid. In Figure 5, we expected an increase in absorbance at 260 to indicate degraded nucleic acid; however the absorbance was unexpectedly decreased near the 230 end of the graph. The second dialysis also showed a decrease in absorbance at 250 and 270 without an improvement in the ratio. This reduction in absorbance without improvement in ratio warranted a native polyacrylamide gel to check if the hexamer of interest was lost through dialysis. The gel

indicated bands with a light smear around the 50 kDa mark; however the relative concentrations of the protein seemed equal. The hexamer is 42 kDa in MW and had appropriately migrated into the gel relative to the protein ladder. The Hfq P protein sample still needs more purification due to its poor 270:250 ratio.

Discussion:

Our hypothesis and assumptions in isolating the *B. anthracis* Hfq P hexamer were incorrect. Following our hypothesis, isolation procedure used on *B. anthracis* Hfq P was nearly identical to *E. coli* Hfq isolation procedure. In the centrifugation step to remove DTT and reduce volume, the *E. coli* Hfq was retained; however the *B. anthracis* Hfq P was not. We believe the *B. anthracis* Hfq P broke into monomer form and went through both the 10 kDa and 5 kDa MW filters. We believe this difference in stability lies in the *E. coli* Hfq's C-terminal tails (accounts largely for the difference in MW between the *E. coli* Hfq and the *B. anthracis* Hfq P). Due to this weakness in stability for the *B. anthracis* Hfq P, dialysis and lyophilization was done as a replacement to centrifugation.

Spectrophotometer reading indicated nucleic acid contamination in both *E. coli* Hfq and *B. anthracis* Hfq P. After nuclease digestion and removal of degraded nucleic acid, the spectrophotometer reading of *E. coli* Hfq showed improved 270/250 ratio. However, the *B. anthracis* Hfq P, with only nuclease digestion, did not show the expected increase in absorbance from degraded nucleic acid. Furthermore, the 2nd dialysis to remove the degraded nucleic acid, reduced the absorbance in both the 250 nm and the 270 nm regions.

Nondenaturing gels for both *E. coli* and *B. anthracis* Hfq samples were performed to check if the protein indicated in the spectrophotometer readings were of the hexamer Hfq. The nondenaturing gel of *E. coli* showed a smear of what we believe to be the aggregates of the

hexamer. Aggregation may be assisted by the C-terminal tails or by protected nucleic acid somehow linking multiple Hfq P together. The *B. anthracis* Hfq P gel showed cleaner bands at the right molecular weights, indicating equal concentrations of Hfq P before and after the second set of dialysis. From the nondenaturing gel of the *B. anthracis*, we calculated the expected absorbance of the *B. anthracis* Hfq P and found that it was much lower than the absorbance the spectrophotometer was showing. Therefore the *B. anthracis* Hfq P sample was still contaminated, but from an unknown source. A procedure to better purify the Hfq P protein needs to be developed.

Despite the failure in purifying the *B. anthracis* Hfq P, one characteristic of the Hfq P against the *E. coli* Hfq was shown. Hfq P appears to be less stable in hexameric form than the *E. coli* Hfq. We believe the reason behind this fragility lies in the lack of C-terminal tails. Future work to provide more evidence to this idea may include a shortened DNA sequence of the *E. coli* Hfq to generate a truncated hexamer protein. The stability of this truncated protein could be tested to observe whether the hexamer stability is influenced by the C-terminal tails. Although this research could not further characterize the Hfq P, there is still value in determining importance of the Hfq P as an RNA chaperone in the bacterial cell. This determination may be useful for future development of drugs targeting Hfq.

Data and Graphs

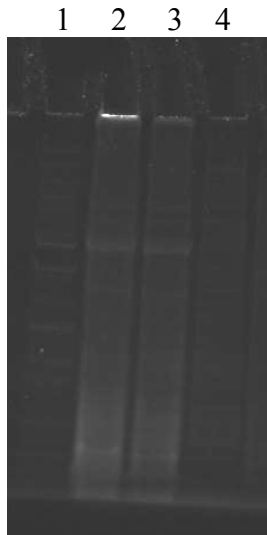


Figure 1: Sypro Orange stained SDS PAGE of samples taken during the *E. coli* Hfq isolation procedure

Lane 1: Protein ladder

Lane 2: Induced ER2566 cells Flask 1

Lane 3: Induced ER2566 cells Flask 2

Lane 4: Flowthrough of Induced ER2566 cell extract

E. coli Hfq before and after nuclease treatment

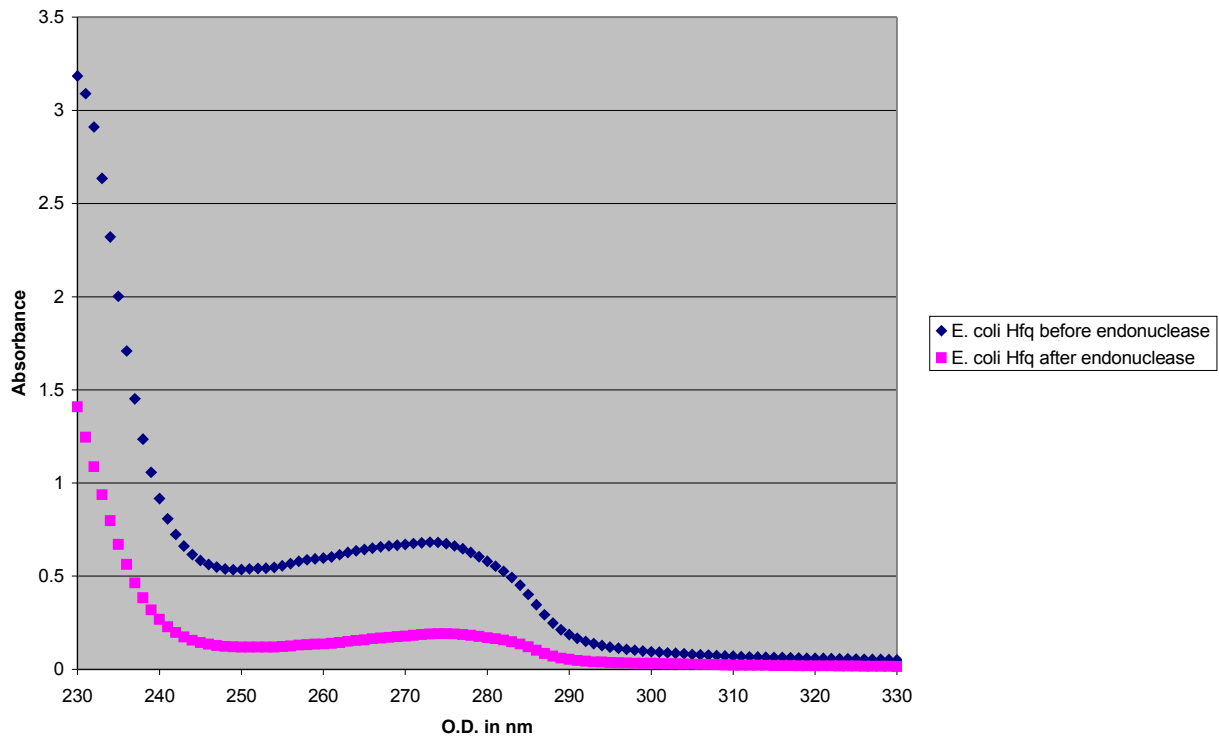


Figure 2: Spectrophotometer readings of *E. coli* Hfq before and after endonuclease treatment. Readings were taken between 230 nm and 330 nm to check for protein and nucleic acid contamination.



Figure 3: Sypro Orange stained native polyacrylamide gel of *E. coli* Hfq hexamer at the 100 kDa MW marker.

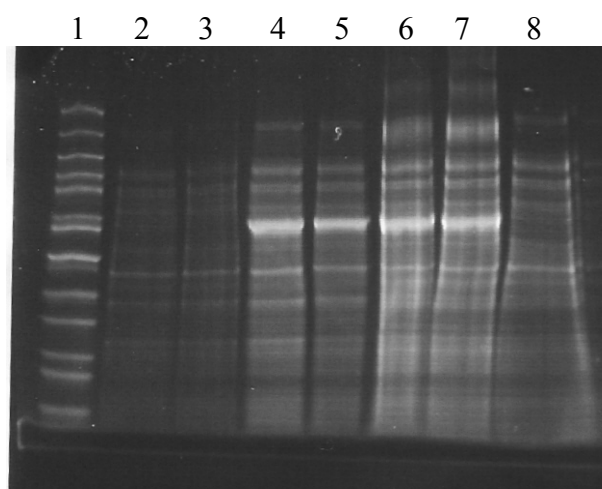


Figure 4: Sypro Orange stained SDS PAGE of samples taken from *B. anthracis* Hfq P's isolation.

Lane 1: Protein ladder

Lane 2: Uninduced ER2566 cells Flask 1

Lane 3: Uninduced ER2566 cells Flask 2

Lane 4: Induced ER2566 cells Flask 1

Lane 5: Induced ER2566 cells Flask 2

Lane 6: Induced ER2566 cell extract for loading onto column Flask 1

Lane 7: Induced ER2566 cell extract for loading onto column Flask 2

Lane 8: Flowthrough of cell extract load (from both flasks)

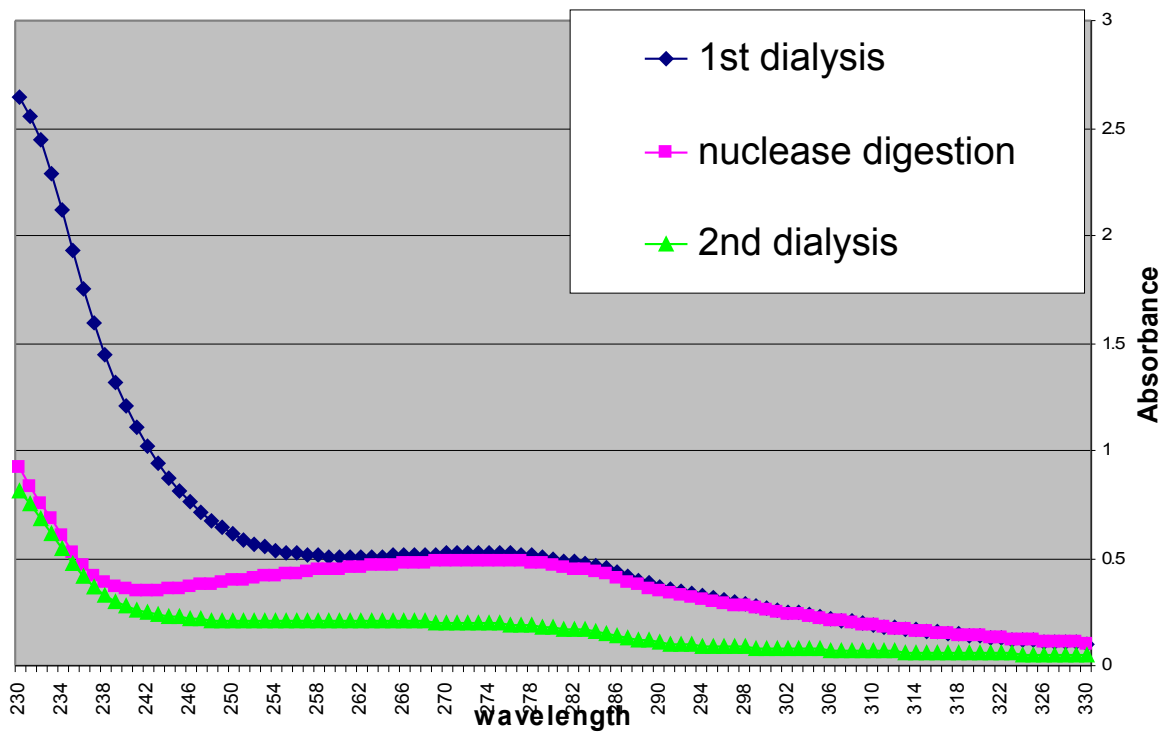


Figure 5: This Absorbance vs. wavelength graph shows three sets of data: top line in blue is the dialysis. Lyophilization step to remove DTT and reduce eluate volume, magenta line shows the Hfq P sample right after endonuclease digestion but before the degraded nucleic acid was cleared by the second set of dialysis. The

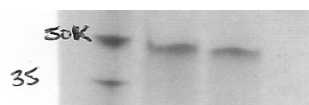


Figure 6: Coomassie Blue stained native polyacrylamide gel with Hfq P after nuclease treatment (lane 2) and after the second set of dialysis (lane 3). Labeled protein ladder in Lane 1 shows the relative molecular weights of the bands.

References

1. Franze de Fernandez, M. T., L. Eoyang, et al. (1968). "Factor fraction required for the synthesis of bacteriophage Q α -RNA," Nature **219**: 588-590.
2. Franze de Fernandez, M. T., W. S. Hayward, et al. (1972). "Bacterial proteins required for replication of phage Q ribonucleic acid. Purification and properties of host factor I, a ribonucleic acid-binding protein." Journal of Biological Chemistry **247**: 824-831.
3. Tsui, H.-C. T., H.-C. E. Leung, et al. (1994). "Characterization of broadly pleiotropic phenotypes caused by an hfq insertion mutation in Escherichia coli K-12." Molecular Microbiology **13**(1): 35-49.
4. Kajitani, M. and A. Ishihama (1991). "IDENTIFICATION AND SEQUENCE DETERMINATION OF THE HOST FACTOR GENE FOR BACTERIOPHAGE Q-BETA." Nucleic Acids Research **19**(5): 1063-1066.
5. Sun, X., I. Zhulin, et al. (2002). "Predicted structure and phyletic distribution of the RNA-binding protein Hfq." Nucleic Acids Research **30**(17): 3662-3671.
6. Valentin-Hansen, Poul, Eriksen, Maiken, and Udesen, Christian (2004). "The bacterial Sm-like protein Hfq: a key player in RNA transactions." Molecular Microbiology **51**(6):1525-1533..
7. Brown, L. and T. Elliott (1996). "Efficient translation of the RpoS sigma factor in Salmonella typhimurium requires host factor I, an RNA-binding protein encoded by the hfq gene." Journal of Bacteriology **178**(13): 3763-3770.
8. Loewen, P. C., B. Hu, et al. (1998). "Regulation in the rpoS regulon of Escherichia coli." Canadian Journal of Microbiology **44**(8): 707-717.
9. Loewen, P. C., and Hengge-Aronis, R (1994). "The role of the Sigma Factor (KatF) in Bacterial Global Regulation." Annual Review of Microbiology **48**: 53-80.
10. Tusi, H.-C. T., G. Feng, et al. (1997). "Negative regulation of mutS and mutH repair gene expression by the Hfq and RpoS global regulators of Escherichia coli K-12." Journal of Bacteriology **179**(23): 7476-7487.
11. Hajnsdorf, E. and P. Regnier (2000). "Host factor Hfq of Escherichia coli stimulates elongation of poly(A) tails by poly(A) polymerase I." Proceedings of the National Academy of Sciences of the United States of America **97**(4): 1501-1505.
12. Brown, L. and T. Elliott (1996). "Efficient translation of the RpoS sigma factor in Salmonella typhimurium requires host factor I, an RNA-binding protein encoded by the hfq gene." Journal of Bacteriology **178**(13): 3763-3770.
13. Sonnleitner, E., S. Hagens, et al. (2003). "Reduced virulence of a hfq mutant of Pseudomonas aeruginosa O1." Microbial Pathogenesis **35**(5): 217-228.
14. Christiansen, J. K., J. S. Nielsen, et al. (2006). "Identification of small Hfq-binding RNAs in Listeria monocytogenes." RNA (Cold Spring Harbor) **12**(7): 1383-1396.
15. Sharma, A. K. and S. M. Payne (2006). "Induction of expression of hfq by DksA is essential for Shigella flexneri virulence." Molecular Microbiology **62**(2): 469-479.
16. Lenz, D., Mok, B. Lilley, et al. (2004). "The Small RNA Chaperon Hfq and Multiple Small RNAs Control Quorum Sensing in Vibrio harveyi and Vibrio cholerae." Cell **118**(1):69-82.
17. Figueroa-Bossi, N., S. Lemire, et al. (2006). "Loss of Hfq activates the sigma(E)-dependent envelope stress response in Salmonella enterica." Molecular Microbiology **62**(3): 838-852.
18. Heidrich, N., A. Chinali, et al. (2006). "The small untranslated RNA SR1 from the Bacillus subtilis genome is involved in the regulation of arginine catabolism." Molecular Microbiology **62**(2): 520-536.